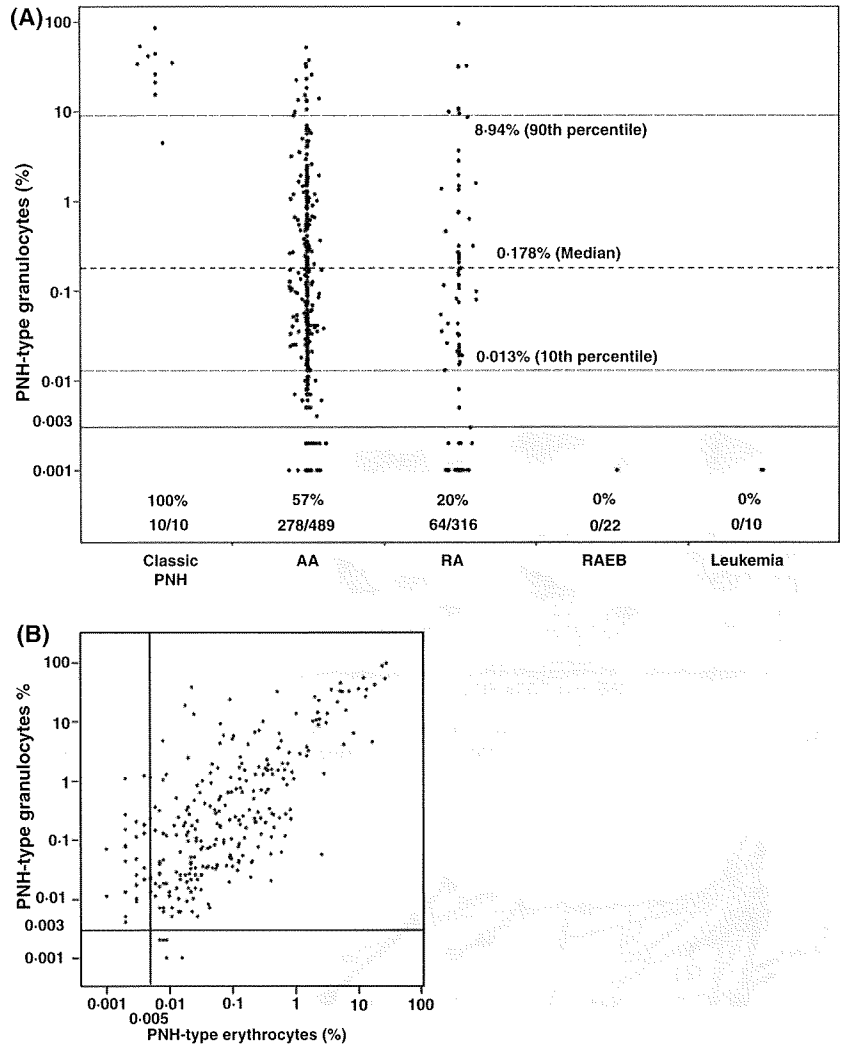


Fig 1. Significant increases in the percentage of PNH-type cells among the patients at the diagnosis of bone marrow failure. (A) PNH-type granulocytes in patients with various diseases. The numbers above the diagnosis represent the prevalence of increased PNH-type granulocytes (%) and the number of patients studied. The solid line denotes a threshold for the significant increase in the PNH-type cell percentage. (B) Correlation between PNH-type granulocytes and erythrocytes.

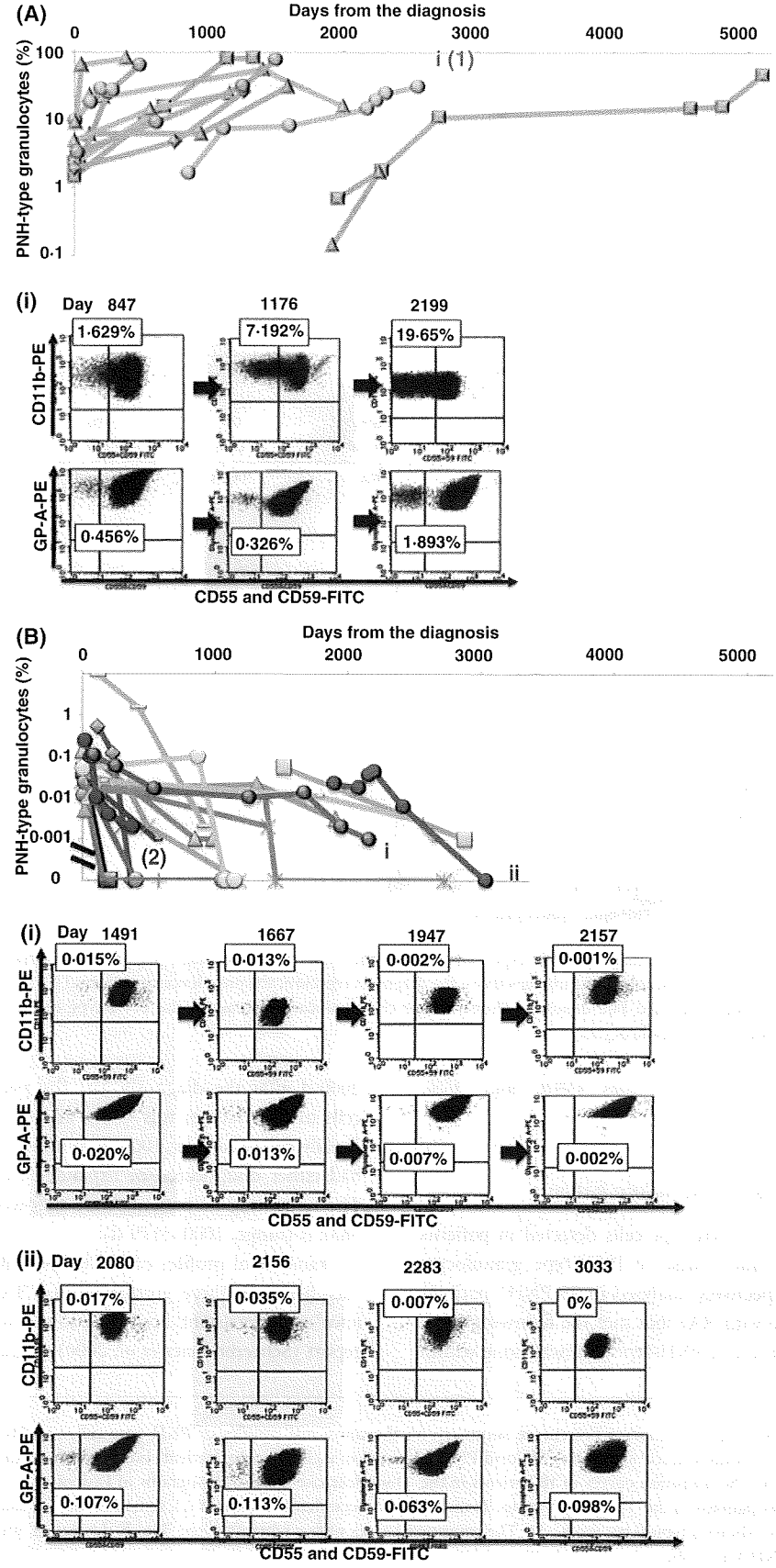
≥21 years than in patients <20 years (59% and 36%, $P = 0{\cdot}004$).

Fate of PNH-type cells in BM failure patients

To clarify the kinetics of PNH-type cells detected in patients with BM failure, the time course of PNH-type granulocyte percentages was retrospectively analysed in 75 PNH^+ patients (72 with AA and three with RA) that met the following three conditions; (i) the first test of PNH-type cells was done within 2000 d after the diagnosis, (ii) the percentage of PNH-type cells in the first test was <10% and (iii) PNH-type cells were serially followed over 1000 d. Within 1000 d, PNH-type cells had either disappeared or expanded to ≥10% in 13 patients. The remaining 62 patients were followed up for a median of 1832 d (range, 1000–3179 d).

Transitional profiles of PNH-type cell percentages could be classified into three groups termed 'Expansion', 'Disappearance' and 'Persistent' according to the terminology of previous report (Schrezenmeier *et al*, 2000). 'Expansion' was defined as

Fig 2. Changes in the PNH-type granulocyte percentage summary on the transional profiles of PNH-type cell. (A) The transition curves of 13 patients in the 'Expansion' group. Clinical symptoms of florid PNH appeared in eight (orange) patients but not in five others (blue). Histograms of Patient 1 (i) are shown. (B) The transition curves of 18 patients in the 'Disappearance' group. Histograms of Patients 1 (i) and 2 (ii) are shown. (C) The transition curves of 44 patients in the 'Persistent' group. Transition curves gradually increased (i), remained flat (ii) and then gradually declined (iii). The patient (iv) curve shows a typical parabola. (D) The transition curves of five patients in the 'Newly developed' group. (E) Summary of the transitional profiles of PNH-type cells.


(A)
Days from the diagnosis
0
1000
2000
3000
4000
5000
PNH-type granulocytes (%)
100
10
1
0·1
i (1)
(i)
Day 847
1176
2199
1·629%
7·192%
19·65%
CD11b-PE
GP-A-PE
0·456%
0·326%
1·893%
CD55 and CD59-FITC
(B)
Days from the diagnosis
0
1000
2000
3000
4000
5000
PNH-type granulocytes (%)
1
0·1
0·01
0·001
0
(2)
i
ii
(i)
Day 1491
1667
1947
2157
0·015%
0·013%
0·002%
0·001%
CD11b-PE
GP-A-PE
0·020%
0·013%
0·007%
0·002%
CD55 and CD59-FITC
(ii)
Day 2080
2156
2283
3033
0·017%
0·035%
0·007%
0%
CD11b-PE
GP-A-PE
0·107%
0·113%
0·063%
0·098%
CD55 and CD59-FITC

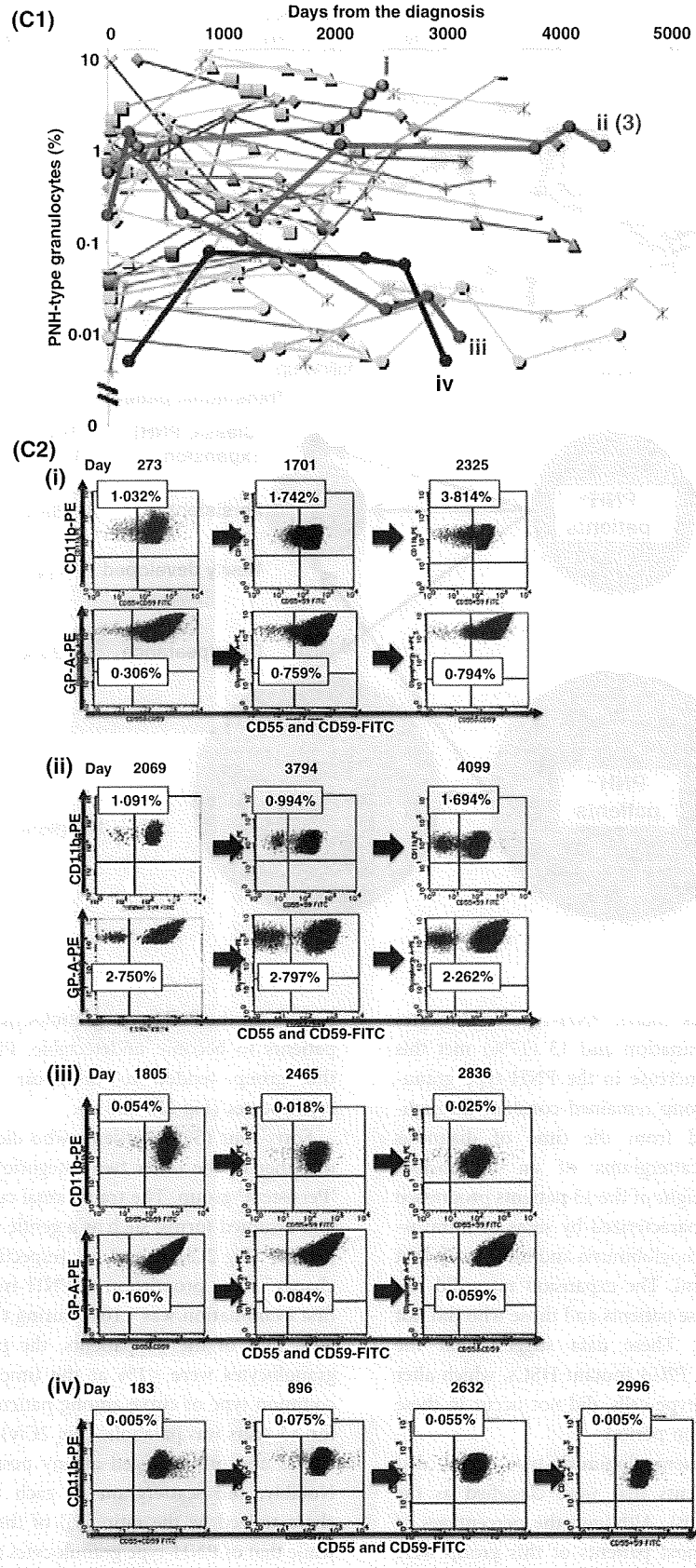


Fig 2. (Continued).

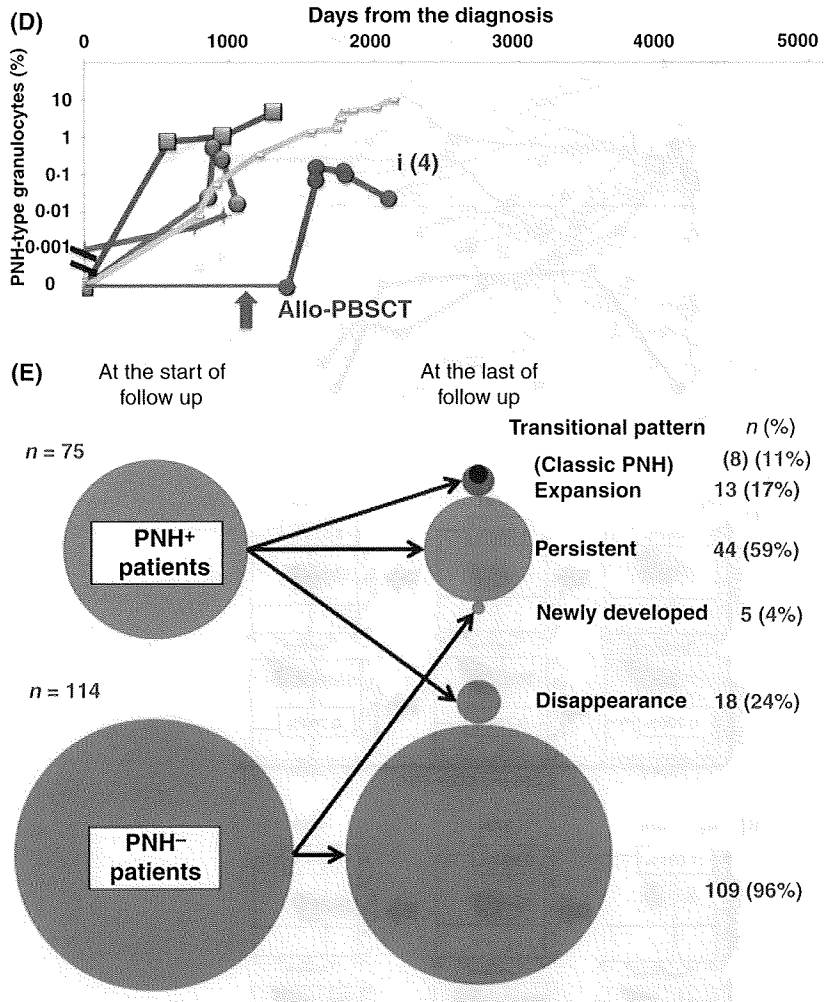


Fig 2. (Continued).

≥10% PNH-type granulocytes and/or PNH-type erythrocytes at the time of the last examination and 13 (17%) met this definition. The rate of the increase in the PNH-type granulocytes in the 'Expansion' group remained constant throughout the observation period from the time of diagnosis (Fig 2A). Representative scattergrams of an 'Expansion' patient is shown in Fig 2Ai. Eight of the 13 patients progressed to classic PNH which was characterized by signs of intravascular haemolysis, such as haemoglobinuria and LDH levels that increased to >1000 i/u per ml. The expansion rates did not apparently differ between these patients and those who did not progress into classic PNH. These data suggest that the secondary genetic changes in *PIGA* mutant HSCs, which alter the expansion rates of PNH-type cells, did not occur in these patients during the observation period.

Paroxysmal nocturnal haemoglobinuria-type cells disappeared in 18 (24%) patients that were classified as the 'Disappearance' group (Fig 2B). Although the percentages of PNH-type granulocytes in most patients of this group were <1% at the time of diagnosis, their PNH-type granulocytes lasted at least 6 months in all patients and it took more than 3 years for the PNH-type granulocytes in 50% (nine) of the 18 patients to become undetectable. PNH-type granulocytes of this group tended to disappear sooner than PNH-type erythrocytes (Fig 2Bi and ii).

Forty-four (59%) patients who did not meet the definition of either of the other two categories were classified into the 'Persistent' group. The transitional curves in this group varied and assumed forms, such as a gentle increase, flat and a gentle decline (Fig 2Ci, ii and iii respectively), and the maximal change in the proportion of PNH-type granulocytes from the first examination was <10% during the observation period. In 80% (35) of the 44 patients, the percentages of PNH-type granulocytes were <1% at the time of diagnosis. The most common type of curve among patients who were followed up for >3 years was parabolic (Fig 2Civ). None of the transitional curves abruptly changed at any point during the follow-up. Notably, the scattergram of each PNH^+ patient that was characterized by the ratio (%) of the type II to type III cells, while that of PNH-type granulocytes to PNH-type erythrocytes was unique and remained essentially unchanged over long periods (Fig 2Ci, ii and iii respectively).

Influence of IST on the fate of PNH-type cells

Forty of the 75 patients were treated with ATG and ciclosporine and 35 (88%) of them responded. The respective proportions of the 'Expansion', 'Disappearance' and 'Persistent' group were 17%, 29% and 54% in 35 responders; 0%, 20% and 80% in five non-responders; and 20%, 20% and 60% in 35 untreated patients. The response rate in each patient group was 100% for the 'Expansion', 91% for the 'Disappearance' and 83% for the 'Persistent' group, suggesting no influence of IST on the fate of PNH-type cells in BM failure patients.

Emergence of PNH-type cells in PNH$^-$ patients

To clarify whether PNH-type cells can emerge in the course of follow-up of PNH$^-$ patients, 114 PNH$^-$ patients (101 with AA and 13 with RA) that met the following three conditions were also analysed; (i) the first test of PNH-type cells was done within 2000 d after the diagnosis, (ii) the percentage of PNH-type cells in the first test was negative and (iii) PNH-type cells were serially followed over 1000 d. The median follow-up period was 1240 d (range, 1010–3101 d). Over time, PNH-type cells became detectable in five patients, who were defined as 'Newly developed' (Fig 2D). The absence of PNH-type cells before PNH$^+$AA developed in one patient (Fig 2Di) was ascertained: the patient had undergone allogeneic PB stem cell transplantation to treat severe PNH$^-$ AA 3 years before developing late graft failure and PNH$^+$ AA affecting donor-derived stem cells was diagnosed because all haematopoietic cells proved to be donor-type. It was the first time that this patient had PNH-type cells detected that were donor-derived. The patient's donor has been haematologically normal and negative for PNH-type cells. The percentage of the donor-derived PNH-type cells reached 0·147% within several months of diagnosis and gently declined over 2 years (Mochizuki *et al*, 2008).

Figure 2E summarizes the transitional profiles of PNH-type cells in PNH$^+$ and PNH$^-$ patients.

PIGA mutations in PNH-type granulocytes among patients with BM failure

To verify the hypothesis that PNH-type cells detectable in patients with BM failure are derived from one or a few primitive HSCs, the *PIGA* gene of PNH-type granulocytes sorted from representative patients in the four different groups designated (1) to (4) in Fig 2A–D was analysed. Two samples were obtained from each patient at intervals of at least 6 months and Table I shows the results of the *PIGA* gene analysis. Only single mutations were detected in the first samples from all four patients at 3/5 to 5/5 frequencies and the same mutations persisted at similar frequencies in the second set of samples, except for Patient 2 in whom the mutation detected in the first sample was not revealed in the second sample. The percentage of PNH-type granulocytes declined to 0·001% when the second sample was obtained.

Discussion

The present study of a large cohort of patients with BM failure confirmed the preliminary findings that an increase in the percentage of PNH-type cells is specific to AA and RA as defined by the FAB criteria (Wang *et al*, 2002). The absence of the increased PNH-type cells in patients with advanced MDS or AML supports the hypothesis that PNH-type cells represent a benign type of BM failure. The increase in the percentage of PNH-type granulocytes was a specific phenomenon to BM failure because it was undetectable in patients with autoimmune diseases that were not associated with BM.

The median percentage of the increased PNH-type granulocytes in PNH$^+$ AA patients at diagnosis was 0·178%, which is 2-log more than the median percentage of PNH-type

Table I. *PIGA* gene mutations of PNH-type granulocytes sorted from four patients.

Patient no.	Age at first analysis (years)	Gender	Day after diagnosis (d)	Interval (d)	Proportion of PNH-type cells (%)	*PIGA* mutation			Frequency of mutation
						Position	Mutation	Consequence	
(1)	76	M	2087	224	8·06	276 bp (exon 2)	Deletion, G	Frameshift	5/5
			2311		24·5				5/5
(2)	83	M	65	251	0·097	505 bp (exon 2)	G to A	Gln to His	3/5
			316		0·001	–	–	–	0/5
(3)	67	M	3924	126	0·774	Splice site (intron 1)	G to A	Splicing abnormality	5/5
			4050		0·723				4/5
(4)	63	M	1602 (268*)	148	0·147	593 bp (exon 2)	Insertion, T	Frameshift	3/5
			1750 (416*)		0·048				5/5

Transitional curves of patients are shown in Fig 3A–D.
*From the day of late graft failure diagnosis.

granulocytes (0·002%) detected in healthy people by Araten *et al* (1999) There may be some concern that this number is too low to be considered a significant increase in the percentage of PNH-type cells. However, our high resolution flow cytometry assay revealed discrete scattergram profiles of granulocytes and erythrocytes in individual patients with PNH-type cells as shown in Fig 2A–C. These profiles persisted for >3 years in most patients regardless of changes in the percentage of PNH-type cells even if the percentages at the time of diagnosis were <1%. Moreover, the correlation between PNH-type granulocytes and erythrocytes was positive in individual patients, including those with <1% PNH-type cells (Fig 1B). These findings indicate that our flow cytometry assay quantifies PNH-type cells accurately at percentages of <1%, which has often been used as a cut-off value in previous studies. Because the PNH-type granulocyte percentage in patients with BM failure shows a log-normal distribution with a median of 0·178%, it therefore seems irrational to establish a cut-off point for a significant increase in the percentage of PNH-type cells at either 1% or 0·1%.

The persistence of a scattergram profile unique to individual patients over long periods of time, even in the 'Disappearance' group indicates that small populations of PNH-type cells are derived from one (or a few) long lived *PIGA* mutated HSCs rather than from *PIGA* mutant HPCs that are short lived. This speculation was substantiated by *PIGA* gene analyses of four patients that revealed only single mutations in sorted PNH-type granulocytes obtained at various time points (Table I). These results were in contrast to previous reports in which multiple *PIGA* mutations were detected in PNH-type granulocytes isolated from AA patients (Mortazavi *et al*, 2003; Okamoto *et al*, 2006). Although the exact reason for these conflicting results is unclear, multiple *PIGA* mutant clones detected by their subcloning method might have picked up *PIGA* mutants, which are thought to be derived from HPCs. Alternatively, our subcloning method may only have detected a predominant clone among several different *PIGA* mutants because a previous study showed that two or more *PIGA* mutant clones are detectable in about 15% of classic PNH, usually consisting of one predominant clone and the other minor clones (Nishimura *et al*, 1999).

The transitional profiles of PNH-type cell percentages present the key clue to understanding the mechanisms of PNH clone expansion. Regardless of the transitional pattern, the curves were essentially linear or parabolic on the semi-logarithmic scale as shown in Fig 2A–C and it looked as though all the transitional profiles started from halfway down the individual curve despite the fact that most patients were examined early after the diagnosis, thus suggesting that the mechanism of the first expansion of PNH-type cells at the initial development of BM failure (first phase), differs from that of PNH-type cell expansion after the development of BM failure (second phase). Figure 3 represents a pattern diagram of the biphasic transitional profiles of PNH-type cells.

The first phase, which occurs before the diagnosis of BM failure, is presumed to represent rapid functional commitment from static to active status of a *PIGA* mutant HSC for haematopoiesis. In most PNH^+ patients, PNH-type cells were detectable from the time of diagnosis, consistent with the preliminary results (Sugimori *et al*, 2006) and those of others based on much less sensitive flow cytometry (Maciejewski *et al*, 2001b; Araten *et al*, 2002); these cells were rarely detectable in the late phase of BM failure in PNH^- patients and such cases were deemed to be exceptional (Fig 2D). Such a commitment of *PIGA* mutant HSCs is probably induced by some immunological mechanisms, due to the fact that the presence of PNH-type cells are associated with good response to IST in AA patients as well as a particular HLA-DR allele (HLA-DR15) (Maciejewski *et al*, 2001a).

The second phase was a continuous change that occurs from several months to years (sometimes a few dozen years) after the first phase. The vector of the transitional curve in this phase was apparently different from that of the first phase, and it remained essentially constant regardless of the response to IST or patient's clinical status; the percentages of PNH-type granulocytes constantly increased and decreased from the time of the first examination in the 'Expansion' and 'Disappearance' groups respectively, thus suggesting the second phase may depend on the proliferation and self-maintenance properties of individual clones that *PIGA* mutant HSC already have at the beginning of this phase rather than on the continuation of the immune selection, which is strongly considered to participate in the first phase.

Although the bigger clone at the beginning of the second phase tended to expand (Fig 2A) while the smaller clone tended to decline (Fig 2B), the great variety of the transitional curve, longevity and size of the PNH-type granulocytes (Fig 2C) indicate theses cells to be derived from *PIGA* mutant clones with various proliferation and self-maintenance properties. Some murine studies have shown considerable variation in the

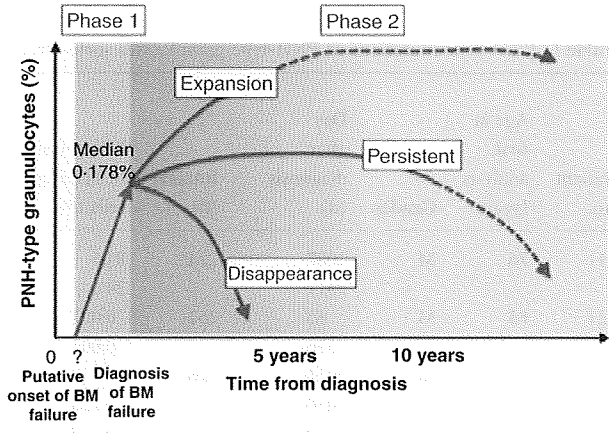


Fig 3. Pattern diagram of the PNH-type cell transition. Phase 1 refers to the first phase which occurs before the diagnosis of BM failure while phase 2 refers to change in the PNH-type cell percentage after the diagnosis. 5Y, 10Y; 5 and 10 years, respectively.

proliferative potential of HSCs defined by a common phenotype (McKenzie *et al*, 2006). Clonal haematopoiesis is detectable in approximately 20% of elderly women (Busque *et al*, 2009) and patients with typical AA in remission, thus indicating that fewer HSCs can support haematopoiesis for long time. All these findings suggest that extensive increase in the percentage of PNH-type cells leading to classic PNH occurs only when an HSC with high proliferative capacity undergoes *PIGA* mutation by chance before the development of BM failure and subsequently gets activated by some sort of immune mechanism(s). On the other hand, a recent study suggested *HMGA2* abnormalities to play a role in the acquisition of proliferative advantage by *PIGA* mutant HSC clones of two patients with classical PNH (Inoue *et al*, 2006). Although the likelihood of HSCs with a *PIGA* mutation undergoing secondary genetic changes is extremely low and the rates of the increase in the PNH-type cell granulocytes in the 'Expansion' group were constant, it is possible that additional gene abnormalities, such as the *HGMA2* mutation, may thus take place in *PIGA* mutant HSCs soon after the development of BM failure.

The results of the present study provide important information for predicting the prognosis of PNH^+ patients. Because others have reported that 10–25% of patients with AA treated with IST progress to classical PNH, those with AA or RA bearing a higher proportion of PNH-type cells at the time of diagnosis are therefore considered to be at high-risk (Tichelli *et al*, 1994; Frickhofen *et al*, 2003). However, this is not necessarily true. A high proportion of PNH-type cells at the time of diagnosis does not always reflect the proliferative potential of *PIGA* mutated HSCs. What determines the risk of progression to classic PNH is a vector of the transition of PNH-type cell percentages during the first 1–2 years after diagnosis. PNH^+ patients with BM failure may therefore not have to worry about the progression to classical PNH if their PNH-type cell percentages do not increase during the first 1–2 years.

In conclusion, PNH-type cells in patients with BM failure are likely to be derived from single *PIGA* mutant HSCs rather than HPCs. The fate of PNH-type cells is therefore composed of two phases, namely the expansion phase due to the selective activation of static *PIGA* mutant HSCs at the onset of immune-mediated BM failure followed by the constant proliferation phase, depending on the self-maintenance and differentiation properties of individual HSC clones. The second phase is not influenced by an ongoing immune attack from which PNH-type cells can escape.

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Author contributions

C.S. and K.M. contributed equally to this work and participated in designing and performing the research. Z.Q. performed some experiments. C.S. and S.N. wrote the paper. C.S., K.M., N.S., K.I., Y.K., H.Y., A.T. and H.O. helped manage the samples. All authors approved the final version of the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Acquired pure red cell aplasia associated with malignant lymphomas: A nationwide cohort study in Japan for the PRCA Collaborative Study Group

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Pure red cell aplasia (PRCA) has been reported in association with lymphoma as one of the autoimmune diseases seen during the course of lymphoid malignancies. However, the relation of PRCA with the underlying lymphomas remains unclear. The aim of this study was to clarify the histologic subtypes of lymphomas, the chronological sequence of anemia and lymphoma, and the response to treatment. We conducted a nationwide survey in Japan. From a cohort of 185 PRCA patients, 8 patients with lymphoma were evaluated. Histologic subtypes varied and the lymphoma was of the B-cell type in four cases and of the T-cell type in four. Four patients simultaneously developed PRCA and lymphoma. Three patients developed PRCA following lymphoma, two of whom developed anemia during remission of lymphoma. PRCA preceded lymphoma in one patient. Effective chemotherapy was associated with remission of anemia in concurrent lymphoma and PRCA. Overall, anemia responded to chemotherapy and/or immunosuppressive therapy in seven patients. In four responding patients, PRCA remained in durable remission without maintenance immunosuppressive therapy, which is different from a recurrent feature of idiopathic PRCA. We suggest that the mechanism of lymphoma-associated PRCA is heterogeneous and that durable maintenance-free remission of anemia can be obtained in some patients. Am. J. Hematol. 84:144–148, 2009. © 2008 Wiley-Liss, Inc.

Introduction

Malignant lymphomas are often accompanied by autoimmune diseases including Sjögren's syndrome, systemic lupus erythematosus and autoimmune hemolytic anemia [1,2]. Lymphomas can also occur resulting from immunosuppressive therapy for autoimmune diseases. Rheumatoid arthritis patients may develop Epstein-Barr virus-associated lymphoma during immunosuppressive therapy such as methotrexate [3].

Pure red cell aplasia (PRCA) has been reported in association with malignant lymphomas as one of the autoimmune diseases often seen during the course of lymphoid malignancies [4]. However, due to the limited number of reported cases of PRCA associated with malignant lymphomas, the relationship between PRCA and the underlying lymphomas remains uncertain, and standard treatment has yet to be established.

The efficacy of immunosuppressive therapy for secondary PRCA could differ among the underlying diseases. We conducted a nationwide survey in Japan between 2004 and 2006 in order to investigate the long-term outcome following immunosuppressive therapy in acquired chronic PRCA in adults. From a cohort of 185 PRCA patients consisting of 73 idiopathic and 112 secondary PRCA cases, we evaluated 8 patients with lymphomas for this report. We also reviewed reported cases of lymphoma-associated PRCA in order to clarify the relation of PRCA to lymphoma.

Results

Histologic subtypes of lymphomas and chronological sequence

Patient age at the onset of PRCA ranged from 47 to 82 years (median age, 68 years) with an equal male to female ratio (Table I). The patient designated as UPN56 has been previously reported elsewhere [5]. Histologic subtypes varied and the lymphoma was of the B-cell type in four cases and of the T-cell type in four. Six of eight patients had advanced disease. One patient developed PRCA four months before the onset of diffuse large B-cell lymphoma (UPN187). In four patients, PRCA and lymphoma simultaneously occurred (UPN166, 18, 57, and 34) (Table I). Three other patients developed PRCA after the onset of lymphoma (UPN56, 128 and 33), in two of whom lymphoma was in complete remission following chemotherapy (Table

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TABLE I. Patient Characteristics

UPN	Age/Sex	Histologic subtypes	Clinical stage	IPI	Days from lymphoma to PRCA	Disease status of lymphoma at the onset of PRCA	Hematological data at the diagnosis of PRCA		
							Hb (g/dl)	Ret (‰)	Erythroid cells in BM (%NCC)
187	76/F	DLBCL	II	L	−114	–	5.7	1.0	0.4
166	75/M	DLBCL	III	L-I	−13	On disease	6.4	0.2	0.2
57	82/F	MZL	IV	L-I	0	On disease	3.0	13.5	4.5
18	62/M	ATLL	IV	H	0	On disease	4.5	0.6	0.2
34	58/F	AILT	IV	H-I	35	On disease	5.4	1.0	0
128	64/M	T-LBL	II	L-I	205	CR	4.8	1.2	Hypoplastic
56	47/M	FL	IV	H-I	720	On disease	6.8	0.1	0.4
33	71/F	AILT	III	H	801	CR	8.1	1.8	4.8

UPN, unique patient number; DLBCL, diffuse large B-cell lymphoma; ATLL, adult T-cell leukemia/lymphoma; MZL, marginal zone lymphoma; AILT, angioimmunoblastic T-cell lymphoma; FL, follicular lymphoma; T-LBL, precursor T lymphoblastic lymphoma; IPI, international prognostic index; L, low; L-I, low-intermediate; H-I, high-intermediate; H, high; Hb, hemoglobin; Ret, reticulocyte; BM, bone marrow; NCC, nucleated cell count.

TABLE II. Induction and Maintenance Therapy for Lymphoma-Associated PRCA

Type of induction therapy for PRCA	UPN	Lymphoma, disease status	Induction for PRCA (dose)[a]	Response of lymphoma	Response of PRCA	Salvage therapy for PRCA (dose) and response	Relapse of anemia	Remission duration for anemia	Maintenance (maintenance-free period)
Chemo	166	DLBCL, on disease	R-CHOP	PR	NR	CsA (150mg), CR	No	33 M	No (27 M)
	34	AILT, on disease	CHOP	CR	CR		No	95 M	No (76 M)
	56	FL, on disease	CHOP	CR	CR		No	126 M	No (127 M)
IST	187	DLBCL, before onset	PSL (40 mg)	N/A	CR	N/A	No	15 M	Yes, PSL 5 mg
	57	MZL	PSL (10 mg)	NR	NR	None	N/A	N/A	N/A
	18	ATLL	CsA (50 mg)	N/A	CR	N/A	No	5 M	Yes, CsA 50 mg
	128	T-LBL, CR	PSL (60 mg)	N/A	PR	N/A	Yes	3 M	No (salvaged by CsA, 97 M for CR2)
	33	AILT, CR	PSL-CsA[b] (60, 200 mg)	N/A	PR/PR	N/A	No	17 M	Yes, PSL 10 mg

[a] The daily doses per body are shown in parenthesis.

[b] Administered in combination later on.

Chemo, chemotherapy; IST, immunosuppressive therapy; R-CHOP, CHOP chemotherapy plus rituximab; PSL, prednisolone; CsA, cyclosporine A; CR, complete response; PR, partial response; NR, no response; N/A, not applicable. M, months.

l). The median hemoglobin concentration at the diagnosis of PRCA was 5.6 g/dl with a range of 3.0 to 8.1 g/dl.

Response of PRCA to chemotherapy and immunosuppressive therapy

Three patients with concurrent PRCA and active lymphoma received chemotherapy as the induction treatment (UPN166, 34, and 56; Table II). In a patient with DLBCL (UPN166), chemotherapy by itself achieved partial response of lymphoma but not response of PRCA. The subsequent administration of cyclosporine achieved CR of PRCA (Table II). In angioimmunoblastic T-cell lymphoma (AILT) and follicular lymphoma patients, chemotherapy alone induced remission for both lymphoma and anemia (UPN34 and 56). All of these three patients remained in CR without maintenance therapy (27, 76, 127 months) (Table II).

Five patients received immunosuppressive therapy as the initial induction treatment of PRCA (UPN187, 57, 18, 128, and 33; Table II). In a patient who developed PRCA before the onset of diffuse large B-cell lymphoma (UPN187), prednisolone was given and resulted in CR. Subsequent lymphoma showed complete response to chemotherapy (CVP). Low dose corticosteroid was given in a patient with splenic marginal zone lymphoma with neither response of lymphoma nor anemia (UPN57). In a patient with adult T-cell leukemia/lymphoma (ATLL) (UPN18), cyclosporine achieved remission of PRCA. The patient then developed an acute crisis and died of disease progression despite of chemotherapy (CHOP). In two patients developing PRCA following successful treatment of lymphoma, corticosteroid was given with partial response (UPN128 and 33). These two patients received chemotherapy with a L10M protocol [6] and a CHOP regimen, respectively. Relapse of anemia was seen in one patient (UPN128) and was salvaged by cyclosporine. This patient was free of PRCA without maintenance immunosuppressive therapy for 97 months.

Taken together, PRCA responded to chemotherapy and/or immunosuppressive therapy in seven of eight patients, and four patients were free of anemia without maintenance immunosuppressive therapy (UPN166, 34, 56 and 128) (Table II). The median time for transfusion-independence from the start of therapy was 95 days with a range of −7 to 450 days.

Outcome of lymphoma-associated PRCA

Four patients were alive, and two of these four remained in remission for both lymphoma and PRCA (Table III). One patient died of infection while lymphoma and PRCA was maintained in remission (UPN33).

Literature search

We found twenty-two reported cases of lymphoma-associated with PRCA and these can be classified into two groups based on the chronological sequence in the onset of lymphoma and anemia. Twelve patients presented with lymphoma and PRCA simultaneously (Table IV) [7–18], 10 patients developed PRCA following lymphoma (Table V) [5,19–27]. Histologic subtypes of lymphoma varied. Three important points can be noted from an extensive review of the literatures. First, effective induction chemotherapy for lymphoma was associated with remission of anemia in patients with concurrent lymphoma and PRCA. Second, a significant fraction of patients were positive for Coombs test. Third, four cases were infected with human parvovirus B19 following chemotherapy including monoclonal antibody (Table V).

TABLE III. Outcome of Lymphoma-Associated PRCA

UPN	Age/sex	Histologic subtypes	Disease status at last observation		Outcome[a]	Cause of death
			Lymphoma	PRCA		
187	76/F	DLBCL	Non-CR	CR	Alive (+17 M)	
166	75/M	DLBCL	Non-CR	CR	Alive (+44 M)	
57	82/F	MZL	Non-CR	Non-CR	Dead (42 M)	Undetermined
18	62/M	ATLL	Non-CR	CR	Dead (8 M)	Disease progression
34	58/F	AILT	CR	CR	Alive (+81 M)	
128	64/M	T-LBL	CR	CR	Alive (+128 M)	
56	48/M	FL	Non-CR	CR	Dead (159 M)	Disease progression
33	71/F	AILT	CR	CR	Dead (45 M)	Infection

[a] Months from the diagnosis of lymphoma.

TABLE IV. Reported Cases of PRCA with Concurrent Lymphoma

Reference	Age/sex	Histologic subtypes of lymphomas	Therapy for lymphomas	Response of lymphoma	Response of anemia to chemotherapy	Others
[7]	16/M	Hodgkin	MOPP	Yes	Yes	
[8]	19/M	Hodgkin	COPP,VEPA	Yes	Yes	
[9]	66/F	Diffuse, mixed	COPP	Yes	Yes	
[10]	61/M	Well-differentiated lymphocytic	COP, Cy, PSL, MACOP-B	Yes	Yes	Coombs (+)
[11]	46/M	AILT	Chemotherapy	Yes	Yes	
[12]	53/M	DLBCL	CHOP	Yes	Yes	Coombs (+)
[13]	54/F	Follicular	R-CHOP	Yes	Yes	Coombs (+)
[14]	71/F	AILT	THP-COP	Yes	Yes	Coombs (+)
[15]	54/F	DLBCL	R-CHOP	Yes	Yes	Coombs (+)
[16]	57/F	Marginal zone	Rituximab	Yes	Yes	
[17]	82/F	DLBCL	Rituximab	Yes	Yes	
[18]	75/F	DLBCL	R-CHOP	Yes	Yes	Coombs (+)

Cy, cyclophosphamide; PSL, prednisolone.

TABLE V. Reported Cases of PRCA with Preceding Lymphoma

Reference	Age/sex	Histologic subtypes of lymphomas	Therapy for lymphomas	Response of lymphoma	Effective therapy for anemia	Others
[19]	25/F	Hodgkin	MOPP/ABV	Yes	CsA, EPO	
[20]	37/F	Hodgkin	Irradiation, MOPP/ABV	Not described	Immunoab, steroid	
[21]	58/M	Follicular	COP	Yes	Spontaneous resolution	
[22]	48/M	Follicular	PROVECIP, BEAC, autologous stem cell transplant	Yes	EPO	
[5]	47/M	Follicular	CHOP, others	Yes	Chemotherapy	
[23]	45/M	Follicular	R-CHOP	Yes	IVIG	Parvo B19
[24]	56/F	Diffuse, mixed	CHOP	Yes	Pred	Coombs (+)
[25]	26/F	DLBCL	R-CHOP	Yes	IVIG	Parvo B19
[26]	56/F	Mycosis fungoides	Alemtuzumab	Not described	IVIG	Parvo B19
[27]	40/F	Follicular	CHOP, R-FND	Yes	IVIG	Parvo B19

PROVECIP, procarbazine, vinblastine and cyclophosphamide, prednisone; R-FND, rituximab, fludarabine, mitoxantrone and dexamethasone; immunoab, immunoabsorbance; CsA, cyclosporine A; EPO, erythropoietin; IVIG, intravenous immunoglobulin.

Discussion

In our patient cohort, immunosuppressive therapy and/or chemotherapy were effective for improving anemia in the majority of patients with lymphoma-associated PRCA. The median time to response after the start of therapy for PRCA was 85 days, which is similar to that of idiopathic PRCA [28]. Wöhrer et al. have reported that rituximab-combined CHOP therapy given for lymphoma treatment is effective for therapy of concurrent rheumatic diseases in non-Hodgkin's lymphoma [29]. Since chemotherapy for malignant lymphoma is immunosuppressive to some extent, the efficacy of chemotherapeutic agents in PRCA patients is not surprising.

However, it is intriguing that four of seven patients maintained remission of anemia without immunosuppressive therapy, which differs from the other form of PRCA [28,30–32]. We have recently reported that discontinuing maintenance immunosuppressive therapy was strongly correlated with relapse of anemia in acquired primary idiopathic PRCA [28,33], and that cyclosporine was quite effective in thymoma-associated PRCA but most patients were receiving maintenance therapy [31]. These results suggest that durable maintenance-free remission of anemia may be obtained in lymphoma-associated PRCA and that PRCA may occur as paraneoplastic syndrome of lymphoid malignancy.

From the point of view regarding the pathogenesis of lymphoma-associated PRCA, it is interesting that positive Coombs test is often associated with PRCA, which suggests the role of autoreactive antibody in lymphoma-associated PRCA. Hauswirth et al. have analyzed more than 100 reported cases in the literatures of non-CLL non-Hodgkin's lymphoma associated with autoimmune hemolytic anemia (AIHA) or Evans' syndrome, and they have reported that warm antibody mediated AIHA was more frequent in B-cell lymphomas, while cold antibody mediated AIHA predominantly occurred in T-cell lymphomas [34]. Some reports demonstrated that serum immunoglobulin showed an inhibitory activity against erythropoiesis in vitro [7,9,21]. Theoretically, the pathogenesis of PRCA in lymphoma patients can

be explained by three potential mechanisms. First, PRCA may occur as a paraneoplastic syndrome of lymphoid malignancy, as described above. Malignant B cell clone might produce autoreactive antibody against erythroid progenitors, and neoplastic T cells might directly or indirectly inhibit erythroid differentiation of hematopoietic stem cells. Cytotoxic therapy may eliminate these pathogenic clones. Second, cytotoxic therapy may cause profound immunosuppression sufficient to inhibit host immunity against parvovirus B19 infection, although our patient cohort did not include this type of patients. In immunocompromised hosts such as recipients of organ transplantation or patients infected with human immunodeficiency virus (HIV) [35–37], acute or chronic anemia can be developed following B19 infection due to the lack of the production of specific antibodies. Third, some patients may develop PRCA via autoimmune mechanisms irrelevant of lymphoma itself following successful chemotherapy, which is supported by the efficacy of immunosuppressive therapy for anemia (UPN128, 33).

One crucial question is how much risk of developing PRCA is present in lymphoma patients. In order to address this issue, it is necessary to establish the cohort of the patients with lymphomas and estimate the risk of developing PRCA. Unfortunately, however, the registry data of lymphoma patients are not available at present in Japan, and thus we need to establish the cancer registration system suitable for this type of analysis.

In conclusion, we have elucidated that the maintenance-free remission is achievable in some PRCA patients associated with lymphomas. Chemotherapy should be introduced for patients with coexisting lymphoma and PRCA. Additional immunosuppressive therapy may be necessary for PRCA that has failed to respond to chemotherapy. However, physicians should be also cautious about unexplained anemia during chemotherapy, combined with monoclonal antibody in particular, because this anemia might be due to persistent parvovirus B19 infection which is treatable with intravenous immunoglobulin [35].

Methods

Collection of the data and patient characteristics. The first questionnaires were sent to 109 institutions in Japan to estimate the number of patients aged 15 and above who had been newly diagnosed as having acquired PRCA between 1990 and 2006. The diagnosis of PRCA was based on the absence of erythroid cells in the bone marrow and the absence of circulating reticulocytes. Morphological diagnosis of bone marrow was done by hematologists at each institution. Human parvovirus B19 infection-associated PRCA was excluded, because the initial aim of the present study was to evaluate the efficacy of immunosuppressive therapy for acquired chronic PRCA in adults. Eligible patients were limited to those who had been diagnosed during the designated period in order to minimize the effect of transfusion-associated hepatitis C virus infection. Overall, 273 patients were enrolled from 45 institutions. Secondary questionnaires were then sent to these institutions to collect data regarding underlying diseases, laboratory findings including peripheral blood cell counts and leukocyte differentials, bone marrow examination, immunologic and cytogenetic parameters, efficacy of immunosuppressive therapy and outcome. A total of 185 patients were enrolled in response to the second questionnaires. Of 185 collected patients, 73 patients were classified as having idiopathic PRCA and 112 patients as having secondary PRCA.

The classification of PRCA was based on the criteria proposed by the Hematopoietic Organs Research Committee of the Ministry of Health, Labor and Welfare of Japan in 2005 [28]. This classification was fundamentally based on the criteria proposed by Dessypris and Lipton [4]. Eight patients demonstrated both malignant lymphoma and PRCA. Personal information was protected by giving each data set a unique patient number at each participating institution. This study was approved by the institutional review board, and performed according to the Declaration of Helsinki and the Ethical Guidelines for Epidemiological Research of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Definition of the response and data analysis. Complete response (CR), partial response (PR), and no response (NR) were defined as the achievement of normal hemoglobin levels without transfusion, the presence of anemia but without transfusion dependence, and the continued presence of transfusion-dependence, respectively [28]. The date of remission was defined as that of the final transfusion after the initiation of remission induction therapy. The minimum period required for evaluation of response to agents was defined as two weeks; therefore, agents added within a two-week period were included into a simultaneous combination with the preceding agents. The agents for remission induction and salvage therapy were defined as those used initially and those used either sequentially or in later combination, respectively. The agent for maintenance therapy was defined as that used or tapered off after successful remission induction. Relapse was defined as the reappearance of transfusion requirement.

Literature search. National Library of Medicine's search service (PubMed) was used to find reported cases of PRCA accompanied by lymphoma published in the literature. The reports that described the outcome of both lymphoma and PRCA were included in this study. Small lymphocytic lymphoma was excluded because this type of lymphoma is often indistinguishable from chronic lymphocytic leukemia, which is well known to be associated with autoimmune hemolytic anemia, immune thrombocytopenia and PRCA [38].

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Appendix

The following institutions participated in the Collaborative Study Group: Aichi Medical School, Akita University, Asahikawa Medical School, Chiba University, Dokkyo Medical School, Ehime University, Fujita Health University, Fukui University, Fukui National Hospital, Fukuoka University, Fukushima Medical University, Gifu University, Gunma University, Hamamatsu Medical School, Hirosaki University, Hiroshima University, Hokkaido University, Hyogo Medical University, Iwate Medical School, Jichi Medical School, Jikei University, Juntendo University, Kagawa Childrens' Hospital, Kagawa University, Kagoshima University, Kanazawa University, Kanazawa Medical School, Kansai Medical University, Kawasaki Medical School, Keio University, Kinki University, Kitazato University, Kobe University, Kochi University, Kumamoto University, Kurume University, Kyoto Prefectural University, Kyoto University, Kumamoto Medical Center, Kyushu University, Mie University, Nagasaki University, Nagoya City University, Nagoya Medical Center, Nagoya University, Nara Medical University, National Cancer Center, National Institute of Infectious Diseases, Niigata University, Nishi Sapporo National Hospital, Nippon Medical School, Nippon University, NTT Kanto Medical Center, Oita University, Okayama Medical Center, Okayama University, Osaka City University, Osaka Medical School, Osaka National Hospital, Osaka University, Ryukyu University, Saga University, Saitama Medical School, Sapporo Medical School, Sendai Medical Center, Shimane University, Shinsyu University, Showa University, St. Marianna University, Teikyo University, Toho University, Tohoku University, Tokai University, Tokushima University, Tottori University, Tokyo Medical Center, Tokyo Medical School, Tokyo Medical and Dental University, Tokyo University, Tokyo Women's Medical School, Tsukuba University, University of Occupational and Environmental Health, Wakayama Medical University, Waseda University, Yamagata University, Yamaguchi University, Yamanashi University, and Yokohama City University.

bjh research paper

NKG2D-mediated immunity underlying paroxysmal nocturnal haemoglobinuria and related bone marrow failure syndromes

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Summary

It is considered that a similar immune mechanism acts in the pathogenesis of bone marrow (BM) failure in paroxysmal nocturnal haemoglobinuria (PNH) and its related disorders, such as aplastic anaemia (AA) and myelodysplastic syndromes (MDS). However, the molecular events in immune-mediated marrow injury have not been elucidated. We recently reported an abnormal expression of stress-inducible NKG2D (natural-killer group 2, member D) ligands, such as ULBP (UL16-binding protein) and MICA/B (major histocompatibility complex class I chain-related molecules A/B), on granulocytes in some PNH patients and the granulocyte killing by autologous lymphocytes *in vitro*. The present study found that the expression of NKG2D ligands was common to both granulocytes and BM cells of patients with PNH, AA, and MDS, indicating their exposure to some incitement to induce the ligands. The haematopoietic colony formation *in vitro* by the patients' marrow cells significantly improved when their BM cells were pretreated with antibodies against NKG2D receptor, suggesting that the antibodies rescued haematopoietic cells expressing NKG2D ligands from damage by autologous lymphocytes with NKG2D. Clinical courses of patients with PNH and AA showed a close association of the expression of NKG2D ligands with BM failure and a favourable response to immunosuppressive therapy. We therefore propose that NKG2D-mediated immunity may underlie the BM failure in PNH and its-related marrow diseases.

Keywords: paroxysmal nocturnal haemoglobinuria, bone marrow failure, NKG2D, ULBP, MICA/B.

Paroxysmal nocturnal haemoglobinuria (PNH) develops by clonal expansion of haematopoietic stem cells that carry acquired *PIGA* mutations (Parker & Ware, 2003). PNH clones bearing *PIGA* mutations neither generate glycosylphosphatidylinositol (GPI) nor express GPI-linked membrane proteins, including complement-regulatory proteins, such as decay-accelerating factor (DAF) and CD59, leading to complement-mediated haemolysis and thrombosis. PNH patients also have various degrees of bone marrow (BM) failure, which is a major cause of death and is essential for clonal expansion of *PIGA* mutant cells (Young, 2000; Nakakuma & Kawaguchi, 2003; Parker & Ware, 2003). It has been suggested that immune-mediated BM damage is a common feature in PNH and its related marrow diseases, such as aplastic anaemia (AA) and hypoplastic myelodysplastic syndromes (MDS) (Young, 2000). Indeed, the BM diseases often show a favourable response to immunosuppressive therapy (IST) (Nakao *et al*, 1994; Horikawa *et al*, 1996; Young, 2000). Natural killer (NK), NKT, and T cells are feasible candidates for effector lymphocytes that damage BM cells, although the most pathognomonic lymphocytes have not been determined yet (Karadimitris *et al*, 2000; Young, 2000; Risitano *et al*, 2002, 2005; Coluzzi *et al*, 2004; Kawaguchi & Nakakuma, 2007). Immune effector cells induce apoptosis of haematopoietic cells (Horikawa *et al*, 1997; Chen *et al*, 2000, 2002; Young, 2000); nevertheless, the molecules that trigger immune attack on haematopoietic cells are unidentified in PNH, in contrast to the various candidates, including heat shock protein-72, in AA (Kawaguchi & Nakakuma, 2007). Target molecules common to PNH, AA, and MDS are also not yet known.

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We have recently reported that stress-inducible membrane proteins, such as cytomegalovirus glycoprotein UL-16-binding proteins (ULBPs) and major histocompatibility complex (MHC) class I chain-related peptides A (MICA) and B (MICB) are expressed on both peripheral blood granulocytes and $CD34^+$ BM cells in some patients with PNH but are not expressed on those cells of healthy individuals except for ULBP3, which is expressed on $CD34^+$ cells (Hanaoka *et al*, 2006). We also found that granulocytes that express stress-inducible membrane proteins are injured *in vitro* by autologous lymphocytes, whereas granulocytes that do not express the membrane proteins are not injured (Hanaoka *et al*, 2006). These findings prompted us to investigate the correlation between the expression of stress-inducible membrane proteins on haematopoietic cells and BM failure. MICA/B and ULBPs are respectively, peptide-anchored and GPI-anchored membrane proteins that are induced when cells are exposed to stress, such as infection and transformation (Bauer *et al*, 1999; Cosman *et al*, 2001; Jamieson *et al*, 2002; Raulet, 2003; Salih *et al*, 2003; Hanaoka *et al*, 2006; Vilarinho *et al*, 2007). The stress-inducible membrane proteins share natural-killer group 2, member D (NKG2D) as a common receptor expressed on lymphocytes including NK, NKT, $CD8^+$ αβ T, γδ T-cells, and a small subset of $CD4^+$ T-cells (Bauer *et al*, 1999; Cosman *et al*, 2001; Jamieson *et al*, 2002; Raulet, 2003; Vilarinho *et al*, 2007). Both MICA/B and ULBPs are then termed NKG2D ligands. Abnormal cells that express NKG2D ligands (except ULBP3) are readily identified and eliminated by $NKG2D^+$ lymphocytes. This study attempted to find the pathological significance of NKG2D ligand expression on blood cells of patients with PNH and PNH-related BM failure syndromes including AA and MDS.

Methods

Patients

The study was approved by the Institutional Review Board and informed consent was obtained. Heparinized peripheral blood samples were collected from 88 patients with BM failure syndromes [19 PNH, 47 AA, 22 refractory anaemia (RA) of MDS] and 17 healthy volunteers. BM aspirates were also obtained from 15 patients (five PNH, seven AA, and three RA). PNH, AA, and RA were diagnosed on the basis of clinical and laboratory evidence of both Coombs negative intravascular haemolysis and the presence of ≥1% GPI-negative erythrocytes (Parker *et al*, 2005; Hanaoka *et al*, 2006), AA and agranulocytosis criteria (Young, 2000), the French- American-British (Bennett *et al*, 1982), and World Health Organization(Jaffe *et al*, 2001) classifications. The PNH patient group comprised 18 classic PNH and one PNH/AA (AA-PNH syndrome). The median percentage of PNH granulocytes (negative for DAF and CD59) of the PNH patients was 72% (range, 1–99%). The clinical and haematological profiles of the patients are summarised in Table I. One, 12, and 7 patients with PNH, AA and RA respectively, were examined at initial diagnosis before treatment, whereas the others were examined at various points during their clinical course. Donors had no symptoms of transformation or infection at the time of examination.

Table I. Patient profiles.

	PNH	AA	RA
Patients	19	47	22
Female/Male	10/9	21/26	4/18
Age: median (range)	56 (24–81)	68 (20–81)	62 (14–83)
Years after initial diagnosis: median (range)	8 (0–30)	2 (0–20)	1 (0–12)
Patients with immunosuppressive therapy	10 (53%)	29 (62%)	2 (9%)
Transfusion-dependent Patients*	6 (32%)	14 (30%)	6 (27%)
Granulocytes: median (range, × 10^9/l)	1·7 (0·2–3·6)	2·0 (0·1–4·9)	1·6 (0·1–6·1)
Haemoglobin: median (range, g/l)	92 (56–133)	87 (36–162)	81 (33–152)
Platelets: median (range, × 10^9/l)	114 (5–296)	40 (2–219)	62 (1–244)
Patients with GPI^- erythrocytes (≥0·1%)			
<1%	0 (0%)	22 (47%)	5 (24%)
≥1%	19 (100%)	0 (0%)	0 (0%)

GPI^-: negative for DAF and CD59.
PNH, paroxysmal nocturnal haemoglobinuria; AA, aplastic anaemia; RA, refractory anaemia.
*The patients who required transfusions of erythrocytes (occasionally together with platelets) at least once per month.

Cell preparation

Peripheral blood granulocytes were isolated using dextran sulphate (Nacalai tesque, Kyoto, Japan) and Ficoll-Hypaque (Amersham, Piscataway, NJ, USA) according to methods described elsewhere (Horikawa *et al*, 1997; Hanaoka *et al*, 2006). Erythrocytes were enriched by depletion of leucocytes and platelets with dextran sulphate. BM mononuclear cells were prepared by Ficoll-Hypaque gradient centrifugation (Hanaoka *et al*, 2006). The viability and purity of isolated cells exceeded 98% as determined by Trypan blue dye exclusion and May-Giemsa staining, respectively.

Antibodies

Mouse monoclonal antibodies (mAbs) to ULBP1 [170818, immunoglobulin (Ig) G2a], ULBP2 (165903, IgG2a), ULBP3 (166510, IgG2a), MICA (159227, IgG2b), MICB (236511, IgG2a), and NKG2D (149810, IgG1) were purchased from R&D systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-conjugated mAb to DAF (JS11KSC2·3, IgG1), phycoerythrin (PE)-texas red (ECD)-conjugated mAb to CD34 (581, IgG1), and rhodamine (RD)1-conjugated mAb to CD2

(T11, IgG1) were purchased from Beckman Coulter (Fullerton, CA, USA). PE-conjugated mAbs to CD11b (Bear1, IgG1) and glycophorin A (GA-R2, IgG2b) were from BD Pharmingen (San Jose, CA, USA).

PE-conjugated mAb to DAF (67, IgG1) was from Caltag Laboratories (Burlingame, CA, USA). FITC-conjugated mAb to CD59 (5H8, IgG1) was donated by M. Tomita of Showa University. PE- and FITC-conjugated affinity purified goat antibodies to mouse IgG were purchased from Chemicon (Temecula, CA, USA) and Zymed Laboratories (San Francisco, CA, USA), respectively. Mouse IgG1 (unlabelled, and labelled with PE, FITC, and ECD), IgG2a (unlabelled), and IgG2b (unlabelled, and labelled with PE) (Beckman Coulter) were used as isotype-matched control immunoglobulins.

Flow cytometry

Cell surface expression was analyzed by flow cytometry as described previously (Horikawa *et al*, 1997; Hanaoka *et al*, 2006). For the analysis of NKG2D ligand expression, cells were incubated with either mAbs to the ligands (ULBP1, ULBP2, ULBP3, MICA, and MICB) or the isotype-matched control immunoglobulin and were then labelled with PE-conjugated goat anti-mouse IgG. If necessary, the cells were labelled with FITC-conjugated mAb to CD59. The BM cells were further labelled with ECD-conjugated mAb against CD34 (a stem cell marker). Cell-surface expression of NKG2D was analyzed by two-colour flow cytometry after labelling with mAb to NKG2D (or control IgG1), FITC-conjugated anti-mouse IgG, and then RD1-conjugated mAb to CD2. To identify cells with the PNH phenotype, cells were labelled with both PE-conjugated mAb to DAF and FITC-conjugated mAb to CD59. Otherwise, the cells were labelled with FITC-conjugated mAbs to DAF and CD59 and subsequently with PE-conjugated mAb to a lineage marker (CD11b for granulocytes and glycophorin A for erythroid cells). The cells were then analyzed with a flow cytometer (EPICS; Beckman Coulter, Fullerton, CA, USA). The detection sensitivity of GPI cells (negative for both CD59 and DAF) was 0·1%. EXPO32 (Beckman Coulter) software was used to quantify the fluorescence signals and to set the logical electronic-gating parameters.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from granulocytes with TRIzol Reagent (Invitrogen, Carsbad, CA, USA) and converted to cDNA with oligo dT primer using a kit (ReverTra; Toyobo, Osaka, Japan) according to the manufacturer's instructions (Hanaoka *et al*, 2006). Full-length cDNA (741 bp) of *ULBP2*, as representative of the three ULBP genes, was amplified by PCR with 0·5 U recombinant Taq DNA polymerase (ExTaq; Takara, Shiga, Japan), 200 μmol/l dNTPs in a buffer (25 μl; 10 mmol/l Tris–HCl, pH 8·3, 50 mmol/l KCl, 1·5 mmol/l $MgCl_2$, and 0·001% gelatin), and 10 pmol of primers (forward: 5′-CGGAATTCATGGCAGCAGCCGCCGCTACCAA-3′, reverse: 5′-CGCTCGAGTCAGATGCCAGGGAGGATGAAGCAGG-3′) (Cosman *et al*, 2001; Hanaoka *et al*, 2006). After initial denaturation at 95°C for 1 min, DNA amplification was performed with a PCR thermocycler (Astec, Fukuoka, Japan) for 35 cycles; each cycle consisted of denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 3 min. A housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used as a control to ensure equal loading of RNA. The products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

Colony-forming cell assay

NKG2D-mediated marrow damage was assessed by *in vitro* colony formation in the presence or absence of mAb to NKG2D using a human haematopoietic-cell colony-forming kit (MethoCult GF$^+$ H4435; StemCell Technologies, British Columbia, Canada) according to the manufacturer's instructions. Briefly, BM mononuclear cells (2×10^6/ml) from healthy donors and patients with PNH and AA were suspended in Iscove's modified Dulbecco's medium containing 2% foetal bovine serum (StemCell Technologies) with either 25 μg/ml of mAb to NKG2D or mouse IgG1 and were incubated for 4 h at 37°C, 5% CO_2. One aliquot of the cell suspension (6×10^5 cells in 0·3 ml) was mixed with 3 ml of methylcellulose-based media (MethoCult). Another aliquot of the mixture (2×10^5 cells in 1·1 ml) was plated onto a 35 mm dish (StemCell Technologies) in duplicate. After culture for 14–18 d, burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte and macrophage (CFU-GM), and colony-forming unit-granulocyte, erythroid, macrophage, and megakaryocyte (CFU-GEMM) were counted. Major colonies were erythroid.

Results

Abnormal expression of NKG2D ligands on granulocytes and BM cells

Flow cytometry detected ULBP1, ULBP2, ULBP3, and MICA on the membrane of granulocytes in eight (42%), nine (47%), 10 (53%), and 11 (58%) of 19 patients with PNH, respectively (Table II). Expression of MICB was also confirmed in four (44%) of nine PNH patients (Table II). In contrast, none of the ligands were detected on the granulocytes of the 17 healthy individuals. Exceptionally, a very low level of MICA expression was transiently detected in one healthy donor. ULBPs and MICA/B were almost always coincidentally expressed (when assessed as described in the footnotes of Table II). The appearance of NKG2D ligands on the membrane of granulocytes was supported by increased *ULBP2* mRNA expression (Fig 1), which is consistent with our previous report (Hanaoka *et al*, 2006). Next, we analyzed the expression of NKG2D

Table II. Expression of NKG2D ligands on granulocytes (flow cytometry).

NKG2D ligands	PNH (19)	Aplastic anaemia (47)	RA (22)	BFS (88)	Healthy control (17)
ULBP 1	8 (42%)	20 (43%)	4 (18%)	32 (36%)	0
ULBP 2	9 (47%)	20 (43%)	4 (18%)	33 (38%)	0
ULBP 3	10 (53%)	20 (43%)	8 (36%)	38 (43%)	0
MICA	11 (58%)	22 (47%)	7 (32%)	40 (45%)	1 (6%)§
MICB	4 (44%)*	16 (34%)	8 (36%)	28 (36%)*	0
ULBP1-3 or MICA/B (a)†	11 (58%)	28 (60%)	8 (36%)	47 (53%)	1 (6%)§
ULBP1-3 and MICA/B (b)‡	10 (53%)	20 (43%)	6 (27%)	36 (41%)	0
b/a	0·9	0·7	0·8	0·8	0

Each value represents the number of patients with granulocytes positive for NKG2D ligands according to flow cytometry.
PNH, paroxysmal nocturnal haemoglobinuria; AA, aplastic anaemia; RA, refractory anaemia, BFS, bone marrow failure syndromes (PNH, AA and RA).
*Nine out of 19 PNH patients were analyzed.
†Patients with granulocytes expressing one or more NKG2D ligands.
‡Patients with granulocytes expressing both one or more of ULBP1-3 and one or more of MICA/B.
§MICA was detected transiently and weakly in only one of the 17 healthy volunteers.

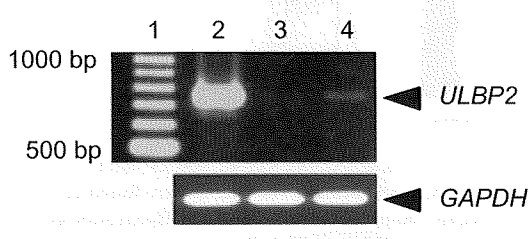

Fig 1. *ULBP2* mRNA expression in PNH. RT-PCR analysis clearly detected *ULBP2* mRNA in the granulocytes of a patient with PNH but not in those of a healthy donor. Lane 1: 100-bp DNA ladders as molecular markers; lane 2: K562 cells positive for *ULBP2* mRNA (Hanaoka *et al*, 2006); lane 3: granulocytes from a healthy donor; lane 4: granulocytes from a patient with PNH who had granulocytes with membrane ULBPs. *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase.

ligands on the granulocytes of patients with PNH-related BM failure syndromes. One or more ligand was detected in 28 (60%) of 47 patients with AA, and 8 (36%) of 22 patients with RA of MDS (Table II). In the 88 patients with BM failure syndromes, 47 (53%) had granulocytes that were positive for one or more NKG2D ligand. Ligands were further detected on BM immature cells in each of the 12 patients who permitted analysis of their BM cells (Table III and Fig 2), whereas the other ligands, except ULBP3, were not detected on the BM cells of healthy donors (Fig 3A), being consistent with our

Table III. Expression of NKG2D ligands on $CD34^+$ BM cells (flow cytometry).

Patient	ULBP1	ULBP2	ULBP3	MICA	MICB
PNH 1	+	+	+	+	ND
PNH 2	+	+	+	+	ND
PNH 3	+	+	+	+	ND
AA 1	+	+	+	+	+
AA 2	+	−	+	−	ND
AA 3	+	+	+	+	ND
AA 4	+	+	+	+	+
AA 5	+	+	+	+	+
AA 6	−	+	+	+	+
RA 1	+	+	+	−	ND
RA 2	−	+	+	+	−
RA 3	+	+	+	+	+

AA, aplastic anaemia, RA, refractory anaemia, ND, not determined.

previous report (Hanaoka *et al*, 2006). $CD34^+$ BM cells from an additional three patients (two patients with PNH and a patient with AA) were also positive for NKG2D ligands, while the $CD34^+$ cells of a healthy donor were negative for the ligands when analyzed by flow cytometry with a mixture of antibodies against ULBP1, ULBP2, MICA, and MICB (cases of AA and healthy control are shown in Fig 3A). ULBP3 was omitted from the BM analysis because only ULBP3 was exclusively expressed on the $CD34^+$ cells of healthy donors in our previous report (Hanaoka *et al*, 2006). The findings show abnormal expression of NKG2D ligands on the peripheral blood and BM cells of patients with the BM failure syndromes of PNH, AA, and RA (MDS).

Recovery of haematopoietic colony formation in the presence of antibodies to NKG2D

We observed abnormal expression of NKG2D ligands on both the granulocytes and BM cells (including $CD34^+$ cells) of some patients with PNH and PNH-related BM failure syndromes (Tables II and III, Figs 1 and 2). In addition, we have previously reported that granulocytes expressing NKG2D ligands are killed by autologous lymphocytes *in vitro* (Hanaoka *et al*, 2006). Therefore, we examined whether the expression of NKG2D ligands on BM progenitor cells impairs haematopoiesis, using an *in vitro* haematopoietic colony formation assay (Fig 3). BM cells were obtained from a patient with AA and two patients with PNH who had BM cells that expressed one or more NKG2D ligands. In the case of AA, the cells were isolated soon after haematopoietic improvement by IST, because the number of BM cells available for the colony assay was insufficient before therapy. Figure 3A shows the expression of NKG2D ligands on the $CD34^+$ cells of this AA patient. $CD34^+$ cells made up 1·6% of the BM cell population. More than half of the patient's $CD34^+$ cells were positive for NKG2D ligands, whereas they were not detected in the $CD34^+$ cells of a healthy control (Fig 3A), indicating abnormal NKG2D ligand

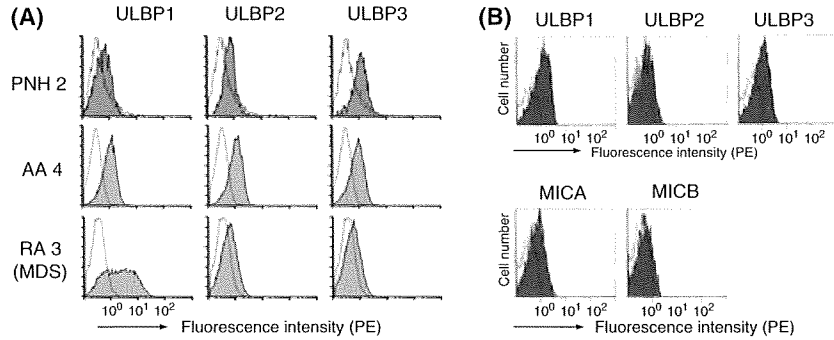

Fig 2. NKG2D ligand expression on $CD34^+$ BM cells. Flow cytometry detected ULBPs [panels (A) and (B)] and MICA/B [panel (B)] (respectively shown in shaded histograms) on the BM cells (labelled with ECD-conjugated mAb to CD34) of patients with bone marrow failure. Patient numbers correspond to those in Table III. The results from one patient in panel (B) (AA 1 in Table III) is also shown in Fig 4A. The percentages of PNH-type BM mononuclear cells of the patients PNH 2, AA 4, and RA 3 in Panel (A) and the patient AA 1 in Panel (B) were 1·9%, 0·2%, 0·5% and 0%, respectively. Dotted lines show nonspecific background staining with isotype-matched control Ig.

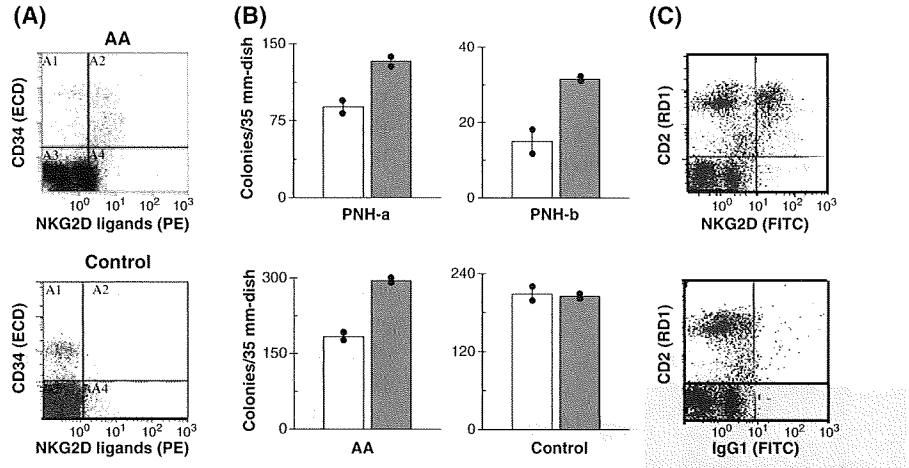

Fig 3. Recovery of haematopoietic colony formation in the presence of antibodies to NKG2D. Panel (A) Two-colour flow cytometry with both an ECD-labelled mAb to CD34 and a mixture of mAbs to NKG2D ligands (ULBP1, ULBP2, MICA, and MICB). The ligands were found on the $CD34^+$ BM cells of a patient with aplastic anaemia (AA), but not on the $CD34^+$ cells of a healthy donor (Control). Panel (B) Haematopoietic colony-formation assays with BM cells from two patients with PNH (PNH-a and PNH-b), a patient with aplastic anaemia [AA: the patient of panel (A)], and a healthy donor (Control). The granulocyte count ($\times 10^9$/l) as a marker of the grade of BM failure was 1·3, 0·4, 1·8, and 2·1 in the patients (PNH-a, PNH-b, and AA) and a healthy control (control), respectively. *In vitro* treatment of BM cells with mAb (25 μg/ml, shaded bars) to NKG2D evidently increased the number of patients' colonies. Open bars: assays with isotype-matched control Ig (mouse IgG1). Each value represents the mean with a range of two data points of duplicate assays. Panel (C) Two-colour flow cytometry of BM cells from the AA patient [the same patient in panels (A) and (B)]. NKG2D was detected on some $CD2^+$ lymphocytes, but not on $CD2^-$ BM cells.

expression in the patient's marrow. Haematopoietic colony formation appeared to be variably impaired in the three patients as compared with that of the healthy control (Fig 3B). Treatment of BM cells with anti-NKG2D mAb dramatically improved the colony formation in all the patients, while no change was observed in the healthy control (Fig 3B). The improvement was evident in the formation of BFU-E and CFU-GEMM (data not shown). Regarding the BM cells of the AA patient, the populations of cells positive for CD2 and cells positive for both CD2 and NKG2D were 13% and 4·9%, respectively (Fig 3C). NKG2D was not detected on $CD2^-$ BM cells containing haematopoietic cells (Fig 3C). Taken together, the findings suggest that the haematopoietic progenitor cells that expressed NKG2D ligands in the patients' BM were damaged by autologous lymphocytes bearing NKG2D.

Correlation of NKG2D ligand expression with BM failure and response to IST

The blood cells of three (two AA and one AA-PNH) patients were prospectively analyzed for more than a year, up to 6 years after initial diagnosis, in order to confirm the positive correlation of NKG2D ligand expression with BM failure, as suggested by the colony assay (Fig 3). Figure 4A shows a representative case of a 48-year-old female patient who had been treated with a low maintenance dose of prednisolone for

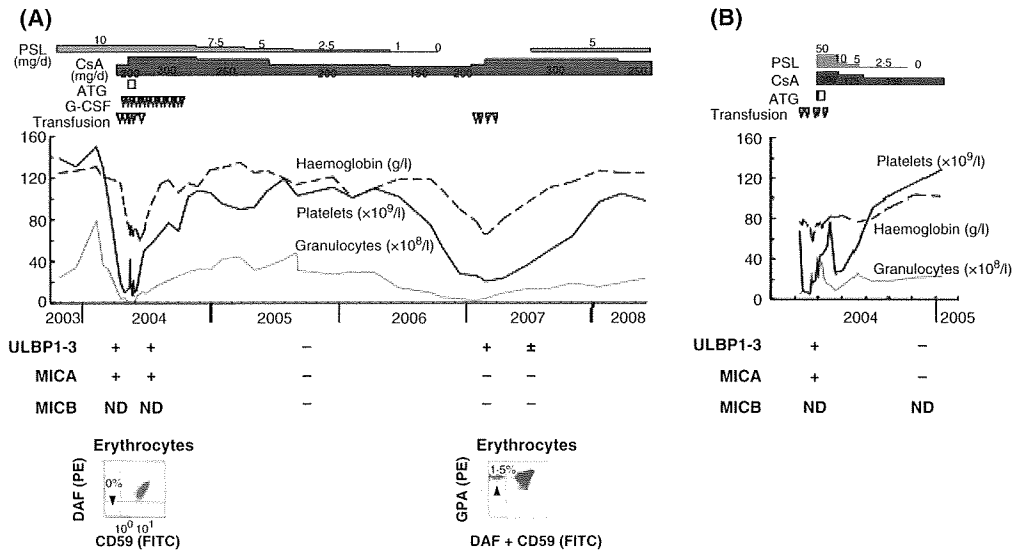

Fig 4. Correlation of NKG2D ligand expression with BM failure and favourable response to IST. Prospective analyses of the clinical courses of a patient with coexisting myasthenia gravis, who developed aplastic anaemia (AA) in 2004 and AA-PNH syndrome in 2007 [panel (A), AA 1 in Table III], and another patient with AA [panel (B)] show a close association between NKG2D ligand expression on granulocytes, pancytopenia, and good response to IST. PSL, prednisolone; CsA, cyclosporine A; ATG, antithymocyte globulin; G-CSF, granulocyte-colony stimulating factor; GPA, glycophorin A; ND, not determined. The arrows indicate PNH-type erythrocytes in the two-colour flow cytometry.

coexisting myasthenia gravis for more than 4 years. In 2004, she developed AA with serious pancytopenia (granulocytes: $0{\cdot}1 \times 10^9$/l, haemoglobin: 64 g/l, platelet count: 7×10^9/l). She had a haplotype of *HLA-DRB1**1501. At the time of the initial diagnosis of AA in 2004, ULBPs 1–3 and MICA were detected on her granulocytes. The ligands were also detected on her $CD34^+$ BM cells (Fig 2B). The BM failure showed a favourable response to granulocyte-colony stimulating factor (G-CSF) plus IST with a combination of cyclosporine A and antithymocyte globulin (ATG). She became transfusion independent and then underwent tapering of IST; however, BM deterioration recurred after 3 years. In contrast to the first episode of BM failure in 2004, the recurrence in 2007 was accompanied by haemolysis (haptoglobin: <74, normal range: 190–1700 mg/l; lactate dehydrogenase: 287 i/u per l, normal range: 112–213 i/u per l) and blood cells negative for DAF and CD59 (cells with the PNH phenotype): 1·5% of glycophorin A $(GPA)^+$ cells (erythrocytes)(Fig 4), 2% of $CD11b^+$ cells (granulocytes), and 8·3% of BM mononuclear cells (data not shown). She was then diagnosed as having PNH with preceding AA (i.e. AA-PNH syndrome) in 2007. Her granulocytes expressed ULBPs again but not MICA/B. The BM failure showed a good response to IST with cyclosporine alone. Thus, analysis of the clinical course shows a close correlation among the expression of NKG2D ligands, BM failure, and successful IST. This correlation was further supported by the analyses of other two patients (an episode of BM failure is shown in Fig 4B).

Discussion

We previously reported not only that NKG2D ligands (ULBP1, ULBP2, ULBP3, MICA, and MICB), which are known to be induced on the membrane of cells that are exposed to stress such as infection and transformation, are expressed on the granulocytes and BM cells of some patients with PNH, but also that the granulocytes that express NKG2D ligands are killed by autologous lymphocytes *in vitro* (Hanaoka *et al*, 2006). The present study confirmed the abnormal expression of NKG2D ligands on both the granulocytes and $CD34^+$ BM cells of additional patients with PNH. Of note, the ligand expression was also observed in patients with PNH-related BM failure syndromes, such as AA and MDS. Namely, one or more of the NKG2D ligands were detected on granulocytes of more than half of the 88 patients with these marrow diseases (Table II) and on the BM cells of all 15 of the patients analyzed (Table III, Figs 2 and 3). Thus, the patients with BM diseases appear to have been exposed to stresses that induced the NKG2D ligands on their peripheral and BM cells.

The lymphocyte-mediated damage of affected (tumour and infected) cells expressing NKG2D ligands (Bauer *et al*, 1999; Cosman *et al*, 2001; Kubin *et al*, 2001; Jamieson *et al*, 2002; Raulet, 2003; Gasser & Raulet, 2006; Vilarinho *et al*, 2007; Vales-Gomez *et al*, 2008) and our previous experience of the killing of granulocytes that express NKG2D ligands by autologous lymphocytes of patients with PNH (Hanaoka *et al*, 2006) prompted us to use a colony assay to determine whether haematopoiesis is impaired by the abnormal expression of NKG2D ligands on the BM progenitor cells of our patients. Beyond our expectation, the BM cells yielded apparently larger number of haematopoietic colonies when formed in the presence of anti-NKG2D mAb *in vitro* (Fig 3B). The BM cells contained lymphocytes positive for both CD2 and NKG2D, but the haematopoietic progenitor cells were negative for NKG2D. It is thought that the anti-NKG2D antibodies

interfered with the engagement between NKG2D on lymphocytes and the NKG2D ligands on haematopoietic progenitor cells and then rescued the progenitors from lymphocyte-mediated damage, allowing the progenitors to form many haematopoietic colonies. The findings not only suggest a critical role for NKG2D-mediated immunity triggered by the appearance of NKG2D ligands in the pathogenesis of BM failure, but also imply a potential remedy for the marrow failure in PNH, AA and MDS via the interruption of NKG2D-mediated immunity. Concerning the NKG2D ligands responsible for marrow failure, ULBP3 is not a candidate because of its discrete features, showing exclusive expression on $CD34^+$ cells of healthy individuals and no engagement in the NKG2D-mediated cytotoxicity (Hanaoka *et al*, 2006).

The pathological expression of NKG2D ligands and the role of NKG2D-mediated immunity in marrow diseases are further supported by our prospective analyses of the clinical courses of three patients with AA and AA-PNH syndrome (Fig 4), which showed a positive correlation of NKG2D ligand expression with marrow failure and successful therapy with immunosuppressants. Of interest, in the clinical course of the patient with AA (Fig 4A), erythrocytes (and granulocytes) that were negative for both DAF and CD59 (a PNH phenotype), which are known as reliable indicators of a favourable response to IST in AA (Sugimori *et al*, 2006), were detectable by our flow cytometry 5 months after the first appearance of NKG2D ligand-expressing granulocytes in 2004 (data not shown). Concerning this latency in detection of PNH-type cells of the patient, it is conceivable that flow cytometric detection needs a period of time for accumulation of PNH-type cells by immunoselection which probably begins with the appearance of NKG2D ligands (Bessler *et al*, 1994; Hertenstein *et al*, 1995; Young, 2000; Murakami *et al*, 2002; Nagakura *et al*, 2002; Inoue *et al*, 2003; Nakakuma & Kawaguchi, 2003; Hanaoka *et al*, 2006; Kawaguchi & Nakakuma, 2007). It is also suggested that detection of PNH clones is occasionally difficult when PNH clones coexist with other expanding clones, like leukaemic cells (Kawano *et al*, 1987). Thus, rather than the detection of PNH-type cells, the detection of NKG2D ligands on blood cells would be of more use for both the early diagnosis of immune-mediated marrow damage and the prediction of successful IST. Also of interest, the granulocytes expressed ULBPs but not MICA/B during the development of PNH in 2007 (Fig 4A). The findings encourage speculation that the PNH clones of the patient expressed neither MICA/B in the pathological environment in 2007 nor ULBPs (an inherent deficiency of GPI-linked proteins as PNH-phenotype) and then acquired a relative survival advantage in the setting of NKG2D-mediated immunity (Nagakura *et al*, 2002; Hanaoka *et al*, 2006), leading to PNH development in 2007.

Here, we propose, for the first time, that NKG2D-mediated immunity, which drives NK, NKT, and T cell activation (Bauer *et al*, 1999; Cosman *et al*, 2001; Jamieson *et al*, 2002; Raulet, 2003; Vilarinho *et al*, 2007), is critically involved, at least in part, in the pathogenesis of PNH and PNH-related BM failure syndromes.

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Authorship and conflict of interest statements

N.H. performed research, analyzed data, and wrote the paper; H.N. and T.K. designed research, analyzed data, and wrote the paper; K.H. and S.N. performed research and analyzed data; Y.T., M.S., K.K., and Y.Y. performed research and analyzed data; all authors checked the final version of the paper. The authors declare no competing financial interests.

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